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(54) Title: THREE-DIMENSIONAL NETWORK FOR BIOMOLECULE DETECTION

(57) Abstract: The present invention provides an apparatus for detecting molecular interactions compatible with electrical and electrochemical detection means. More specifically, the invention provides a bioarray that is fabricated from a porous substrate plated with a conductive layer, more specifically, a porous substrate plated with metal, more specifically, a porous hydrogel media substrate plated with metal.

THREE-DIMENSIONAL NETWORK FOR BIOMOLECULE DETECTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to an apparatus for detecting molecular interactions. In particular, the invention relates to biochip arrays manufactured in part from porous media. More particularly, the invention relates to a biochip array that is fabricated from a porous substrate with metal, metal oxides, metal nitrides, or metal carbides deposited on a porous surface thereof, and even more particularly to a polymeric porous hydrogel substrate deposited on the porous surface with a layer of an electrically-conductive material.

2. Background of the Invention

Microfabricated arrays (biochips) of oligonucleotides, nucleic acids, or peptides have utility in a wide variety of applications, including DNA and RNA sequence analysis, diagnostics of genetic diseases, gene polymorphism studies, analysis of gene expression, and studies of receptor-ligand interactions. In the process of biochip fabrication, large numbers of probe molecules are bound to small, defined regions of a substrate. Glass slides, silicon wafers, or polymeric hydrogels may be used as a biochip substrate, with a two-dimensional or three-dimensional substrate surface utilized for probe attachment. As compared to two-dimensional biomolecule immobilization substrates, three-dimensional immobilization substrates offer an advantage of increased sensitivity. This increased sensitivity results from the larger surface area of three-dimensional substrates, allowing for the immobilization of a greater number of probe molecules in a fixed two-dimensional area, and in turn permitting the interaction of a greater number of bound probe molecules with target molecules (biomolecules) in a given sample. This increased capacity is achieved without an increase in the surface density of attached probes, which is a limitation of two-dimensional array due to probe-probe interactions that inhibit hybridization.

Polymeric hydrogels offer several advantages over both glass and silicon as a substrate material for biochip preparation. One of the primary advantages for using porous hydrogel media over other substrate materials is that the polymeric hydrogel matrix is inherently a three-dimensional porous structure, which eliminates the need to perform lithography and etching to form artificial three-dimensional structures.

While the inherent three-dimensional structure of porous hydrogel media can be advantageous for loading probe molecules such as oligonucleotides, it also poses additional challenges for immobilization chemistry. The use of porous hydrogel media in the fabrication of bioarrays, for example, is restricted by complicated probe attachment chemistries, which often result in inconsistent probe attachment yield, thus increasing the cost of quality control. In addition, since polymeric hydrogels are not electronically conductive, arrays made with such hydrogels are not suitable for electrical or electrochemical detection of molecular interactions, particularly bioarrays as defined herein.

Thus, there remains a need in the art for a method for fabricating porous substrates that facilitate probe attachment. Specifically, there remains a need in the art for a method of producing a porous substrate for use in the fabrication of biochip arrays that is simpler, less expensive, generates a higher yield of attached probes, and permits the use of a wider range of detection methods, including electrical, electrochemical, optical and radiation-based detection technologies.

SUMMARY OF THE INVENTION

The invention provides an apparatus for detecting molecular interactions. In particular, the invention provides a biochip array manufactured in part from porous media. More particularly, the invention provides a biochip array that is fabricated from a porous substrate with a conductive material, *e.g.*, metal or metal oxides, deposited (in some embodiments, plated) on a porous surface thereof. In alternative embodiments, the invention provides a polymeric, porous hydrogel substrate with a conductive material deposited on the porous surface of the hydrogel. In preferred embodiments, the conductive substance is a metal, metal oxide, metal nitride, or metal carbide.

Additionally, the invention provides a three-dimensional media compatible with electrical and/or electrochemical detection techniques including impedance, AC impedance, impedance spectroscopy, cyclic voltammetry, alternating cyclic voltammetry, stripping voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, and combinations thereof, for detecting interactions between molecules, particularly biomolecules.

The apparatus of the present invention can be used to detect molecular interactions between probes immobilized on the porous surface of the porous substrate and target molecules in a sample reaction mixture. Preferred probe molecules include but are not limited to oligonucleotides, nucleic acids, or peptides.

The apparatus of the present invention offers several advantages. One advantage is that the metallic surface of the deposited polymeric hydrogel array permits probe molecules to be attached to the substrate using a simpler reaction chemistry than that required for the attachment of probe molecules to the porous hydrogel media itself. For example, oligonucleotide probe molecules modified by having a thiol group can be attached to a gold-plated polymeric hydrogel array using self-assembly techniques known to those with skill in the art. In addition, the metallic surface of conductive porous substrates of the present invention permits electrical and/or electrochemical detection methods such as impedance, AC impedance, impedance spectroscopy, cyclic voltammetry, alternating cyclic voltammetry, stripping voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, and combinations thereof, to be used for assaying molecular interactions between immobilized probe molecules and biomolecules in a reaction mixture.

The pore size of the porous media can be controlled by varying process conditions such as temperature, monomer and/or crosslinker concentration, concentration of the plating solution and time of conductive material deposition. This is desirable for optimizing diffusion kinetics for biomolecules having different sizes or conformations.

In addition, the hydrogel substrate of the present invention, which is fabricated in part from a three-dimensional substrate, does not require the complex manufacturing steps that are necessary for producing three-dimensional structures from other types of substrate materials.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a scanning electron micrograph (0-300 nm scale) of a hydrogel array following 40 minutes of electroless gold plating.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The apparatus of the present invention is comprised of a solid substrate, a porous media placed on one (preferably, the top) surface of the solid substrate, and a layer of conductive material deposited on a porous surface of the porous media. Most preferably, biological probe molecules are immobilized on the conductive surface of the porous media. In preferred embodiments of the present invention, changes in electrical properties of attached probe-comprising porous media, following exposure to and interaction with complimentary targets comprising a biological sample or reaction mixture (wherein said targets may or may not additionally comprise an electrical or electrochemical reporter group), are detected by electrical means.

Complimentary targets may or may not additionally comprise an optical tag or radiolabel. In other embodiments of the present invention, the apparatus further comprises a means for detection of probe-target interactions wherein the target molecule carries an optical tag or radiolabel.

Most preferably, a plurality of probe molecules are attached to the porous media to provide a bioarray. As used herein, the term "bioarray," "biochip" or "biochip array" refer to an ordered spatial arrangement of immobilized biomolecules or polymeric

anchoring structures on a solid supporting substrate. Preferred probe molecules include nucleic acids, oligonucleotides, peptides, ligands, antibodies and antigens; oligonucleotides are the most preferred probe species.

The solid substrate in the embodiment advantageously can be made of glass, ceramic, plastic, semiconductor wafer such as silicon or gallium-arsenic, or printed circuit board (PCB).

In some embodiments of the present invention, the probes are oligonucleotide probes having a sequence comprising from about 10 to about 100 nucleotide residues, and said probes are attached to the conductive porous surface of the porous media using techniques known to those with skill in the art. In other embodiments, the probes are peptides, such as receptors, ligands, antibodies, antigens, or synthetic peptides, and said probes are attached to the conductive porous surface of the porous media using techniques known to those with skill in the art. In preferred embodiments, the probes are covalently attached to the surface of the pores of the porous substrate.

In some embodiments, the method of the present invention is used to detect single base mismatches within nucleic acid probe-target complexes. In other embodiments, the method of the present invention is used to quantify target molecules in a reaction mixture for gene expression analyses.

The conductive layer in the apparatus in accordance with the present invention may be most advantageously fabricated using electroplating, electroless plating, thermal deposition, or plasma enhanced chemical vapor deposition (PECVD) techniques. In the preferred embodiment of the present invention, the conductive layer is affixed using an electroless plating technique. In the preferred embodiment, the conductive layer is a metal. More preferably, the conductive layer is a porous film of a metal, such as gold, platinum, titanium, or copper, or a metal oxide, a metal nitride, a metal carbide, or carbon (graphite).

The porous media of the present invention may be a conductive or nonconductive polymer. Non-limiting examples of the porous media of the invention include polyacrylamide gel, agarose gel, cellular gel, polyethylene glycol, polypyrrole, carbon, carbides, oxides, nitrides, or other suitable materials known to those with skill in the art. In the preferred embodiment of the present invention the porous media is a

polyacrylamide gel (termed a "hydrogel" herein). The porous hydrogel media of the present invention may be produced using sol-gel, aerogel, or other fabrication techniques known to those with skill in the art.

The porous media of the present invention may be plated or thermally deposited with conductive materials, including metals such as gold, copper, nickel, aluminum, platinum, and silver, metal oxides such as tin oxide, zinc oxide and indium tin oxide, metal nitrides such as nobium nitride, or metal carbides such as tin carbide. In a preferred embodiment of the present invention, the porous media is plated with gold. In some embodiments of the present invention, the porous media is removed from the metal layer by exposure, for example, in a surfactant solution at 100°C.

The metal plating or deposition of conductive material used to fabricate the apparatus of the present invention is preferably biocompatible with the molecular reactions to be performed on the bioarray. In other embodiments of the present invention, the biocompatibility of the plated surface of the porous substrate is enhanced by coating the bioarray with a conformal compound such as parylene.

In one method of the present invention, molecular interactions between an immobilized probe and target molecule are detected by contacting a plurality of probes immobilized onto the surface of the pores of a porous substrate, and wherein a thin conductive layer has been placed in contact with the surface of the porous substrate, with an electrolyte solution, detecting an electrical signal in a plurality of pores of the porous substrate, exposing the porous substrate to a reaction mixture containing a target molecule in order to generate probe-target complexes, and detecting an electrical signal in the pores of the porous substrate. In alternative embodiments of the method of the present invention, target molecules additionally comprise an electrical or electrochemical reporter, optical tag, or radiolabel.

In preferred embodiments, the apparatus of the present invention is used for the electrical and/or electrochemical detection of molecular interactions between immobilized probe molecules and biomolecules in a particular reaction mixture. Electrical and/or electrochemical detection methods including, but not limited to, impedance, AC impedance, impedance spectroscopy, cyclic voltammetry, alternating cyclic voltammetry, stripping voltammetry, pulse voltammetry, square wave

voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, and combinations thereof, can be used with the apparatus of the present invention.

In preferred embodiments of the present invention, the electrical or electrochemical detection method is AC impedance and the AC impedance is measured over a range of frequencies prior to and after exposing the plurality of probes immobilized onto the surface of the pores of the apparatus of the present invention to a reaction mixture containing a target molecule. In some embodiments, AC impedance is measured by transient methods with AC signal perturbation superimposed upon a DC potential applied to an electrochemical cell. In some embodiments, AC impedance is measured by impedance analyzer, lock-in amplifier, AC bridge, AC voltammetry, or combinations thereof.

In some embodiments of the method of the present invention, molecular interactions between probe molecules bound to hydrogel porous microelectrodes and electrochemically-labeled target molecules are detected. Electrochemically-labeled target molecules useful in the methods of the present invention may be prepared by labeling suitable target molecules with any electrochemically-distinctive oxidation/reduction (redox) reporter that does not interfere with the molecular interaction to be detected. In preferred embodiments of the method of the present invention, target molecules are labeled with electrochemical reporter groups comprising a transition metal complex, most preferably containing a transition metal ion that is ruthenium, cobalt, iron, or osmium.

In other embodiments of the present invention, target molecules may be labeled with the following non-limiting examples of electrochemically-active moieties:

Redox moieties useful against an aqueous saturated calomel reference electrode include: 1,4-benzoquinone, ferrocene, tetracyanoquinodimethane, N,N,N',N'-tetramethyl-p-phenylenediamine, or tetrathiafulvalene;

Redox moieties useful against an Ag/AgCl reference electrode include: 9-aminoacridine, acridine orange, aclarubicin, daunomycin, doxorubicin, pirarubicin, ethidium bromide, ethidium monoazide, chlortetracycline,

tetracycline, minocycline, Hoechst 33258, Hoechst 33342, 7-aminoactinomycin D, Chromomycin A_3, mithramycin A, Vinblastine, Rifampicin, $Os(bipyridine)_2(dipyridophenazine)_2^+$, $Co(bipyridine)_3^{3+}$, or Fe-bleomycin.

The electrochemically-active moiety comprising the electrochemically active reporter-labeled target molecule of the method of the present invention is optionally linked to the target molecule through a linker, preferably having a length of from about 10 to about 20 Angstroms. The linker can be an organic moiety such as a hydrocarbon chain $(CH_2)_n$, where n is an integer from about 1 to about 20, or can comprise an ether, ester, carboxyamide, or thioether moiety, or a combination thereof. The linker can also be an inorganic moiety such as siloxane (O-Si-O). The length of the linker is selected so that the electrochemically-active moiety does not interfere with the molecular interaction to be detected.

Electrochemical contact is advantageously provided using an electrolyte solution in contact with each of the hydrogel porous microelectrodes of the invention. Electrolyte solutions useful in the apparatus and methods of the invention include any electrolyte solution at physiologically-relevant ionic strength (equivalent to about 0.15 M NaCl) and neutral pH. Examples of electrolyte solutions useful with the apparatus and methods of the invention include but are not limited to phosphate buffered saline, HEPES buffered solutions, and sodium bicarbonate buffered solutions. In some embodiments of the present invention, the electrolyte solution comprises metal, non-metal, or polymerized cations that are ion-conductive and capable of reacting with probes or probe-target complexes.

The Example that follows is illustrative of specific embodiments of the invention, and various uses thereof. This Example is set forth for explanatory purposes only, and is not to be taken as limiting the invention.

EXAMPLE 1

Electroless Gold Plating of Acrylamide Gel Pad

Polyacrylamide hydrogel arrays were fabricated on glass slides with dimensions of 3 in. by 1 in. The hydrogel array was photopolymerized on the glass slide using

bisacrylamide as cross-linking agent at a final concentration of 5%. The polymerized hydrogel pads had final dimensions of 100 μm by 100 μm, a thickness of 25 μm and a pad to pad distance of 300 μm. The size of the complete array was 28 test sites by 28 test sites. Following preparation, the hydrogel arrays were hydrated in water for one hour. During hydration, electroless plating solution (Oremerse Mn, obtained from Technic Inc., Cranston, RI), containing 0.25 g/gal. of elemental gold, was heated to 65°C and allowed to stabilize at that temperature in a water bath. Hydrogel arrays were immersed in the plating solution for between 1 min. and 60 min. The thickness of the gold plating being applied to the surface of the hydrogel media was controlled by varying the temperature of the water bath, the concentration of the plating solution, and the plating time. By varying these parameters, the physical properties of the plated media, such as pore size and pore distribution, can be varied. For example, by increasing the temperature of the water bath, the pore size of the hydrogel media could be increased during the plating process. Following plating, traces of the plating solution were removed by rinsing the hydrogel arrays in distilled water and the hydrogel arrays were allowed to air dry. Plated hydrogel arrays, and unplated controls, were then examined by scanning electron microscopy (SEM).

Figure 1 illustrates scanning electron micrographs (0-300nm scale) of a hydrogel array following 40 min. of electroless gold plating. After 40 min. of plating in an electroless plating solution as described above, a porous gold matrix was obtained as illustrated by scanning electron micrograph (0-300nm scale) in Figure 1. Oligonucleotide probes modified with a thiol linker are attached to the porous gold matrix using conventional thiol-gold attachment chemistries well known in the art.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WHAT WE CLAIM IS:

1. An apparatus for detecting molecular interactions, comprising:

(a) a porous substrate comprising a plurality of pores having a surface adapted for attaching probe molecules thereto,

(b) a thin conductive layer placed in contact with the surface of the porous substrate, and

(c) a plurality of probes immobilized onto the thin conductive layer.

2. The apparatus of claim 1, wherein the porous substrate is a conductive polymer.

3. The apparatus of claim 2, wherein the conductive polymer is a polypyrrole, oxide, carbide, or carbon.

4. The apparatus of claim 1, wherein the porous substrate is a nonconductive polymer.

5. The apparatus of claim 4, wherein the nonconductive polymer is a polyacrylamide gel, an agarose gel, polyethylene glycol, a cellular gel or a sol-gel.

6. The apparatus of claim 1, wherein the conductive layer is a metal.

7. The apparatus of claim 6, wherein the conductive layer is gold, silver, copper, aluminum, platinum or nickel.

8. The apparatus of claim 1, wherein the conductive layer is a metal oxide.

9. The apparatus of claim 1, wherein the conductive layer is a metal nitride.

10. The apparatus of claim 1, wherein the conductive layer is a metal carbide.

11. The apparatus of claim 1, wherein the thin conductive layer is metal and is placed in contact with the porous substrate by plating the porous substrate with the metal.

12. The apparatus of claim 1, wherein the probes are oligonucleotides.

13. The apparatus of claim 1, wherein the probes are nucleic acids.

14. The apparatus of claim 1, wherein the probes are peptides.

15. The apparatus of claim 1, wherein the probes are attached to the surface of the pores.

16. The apparatus of claim 15, wherein the probes are attached to the surface of the pores using thiol chemistry.

17. The apparatus of claim 1, wherein a molecular interaction between the probe molecules and a target molecule contained in a reaction mixture is detected by contacting the reaction mixture with the plurality of probes attached to the surface of the pores using optical detection methods.

18. The apparatus of claim 17, wherein the target molecules are optically labeled.

19. The apparatus of claim 1, wherein a molecular interaction between the probe molecules and a radiolabeled target molecule contained in a reaction mixture is detected by contacting the reaction mixture with the plurality of probes attached to the surface of the pores using radiolabel detection methods.

20. The apparatus of claim 1, wherein a molecular interaction between the probe molecules and a target molecule contained in a reaction mixture is detected by

contacting the reaction mixture with the plurality of probes attached to the surface of the pores using electrical detection methods.

21. The apparatus of claim 20, wherein the target molecules are labeled with electrochemically-active moieties.

22. A method for the electrical detection of molecular interactions between an immobilized probe and a target molecule, comprising:

(a) contacting a plurality of probes immobilized onto the surface of the pores of a porous substrate, and wherein a thin conductive layer has been placed in contact with the surface of the porous substrate, with an electrolyte solution,

(b) detecting an electrical signal in a plurality of pores of the porous substrate,

(c) exposing the porous substrate to a reaction mixture containing a target molecule in order to generate probe-target complexes, and

(d) detecting an electrical signal in the pores of the porous substrate.

23. The method of Claim 22, wherein the electrolyte solution comprises metal, non-metal or polymerized cations that are ion-conductive and capable of reacting with probes or probe-target complexes.

24. The method of Claim 22, wherein molecular interactions between an immobilized probe and a target molecule are detected by applying an electrical detection method selected from the group of impedance, AC impedance, impedance spectroscopy, cyclic voltammetry, alternating cyclic voltammetry, stripping voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, and combinations thereof.

25. The method of Claim 24, wherein the electrical detection method is AC impedance and the AC impedance is measured over a range of frequencies prior to and

after exposing the plurality of probes immobilized onto the surface of the pores of a porous substrate to a reaction mixture containing a target molecule.

26. The method of Claim 24, wherein the electrical detection method is AC impedance and the AC impedance is measured by transient methods with AC signal perturbation superimposed upon a DC potential applied to an electrochemical cell.

27. The method of Claim 24, wherein the electrical detection method is AC impedance and the AC impedance is measured by impedance analyzer, lock-in amplifier, AC bridge, AC voltammetry, or combinations thereof.

28. The method of Claim 22, wherein the molecular interactions detected thereby are single base mismatches within nucleic acid probe-target complexes.

29. The method of Claim 22, wherein the molecular interaction detected is quantification of target molecules in a reaction mixture for gene expression analyses.

30. A method for the electrochemical detection of molecular interactions between an immobilized probe and an electrochemically active reporter-labeled target molecule, comprising:

(a) contacting a plurality of probes immobilized onto the surface of the pores of a porous substrate, and wherein a thin conductive layer has been placed in contact with the surface of the porous substrate, with an electrolyte solution,

(b) detecting an electrochemical signal in a plurality of pores of the porous substrate,

(c) exposing the porous substrate to a reaction mixture containing an electrochemically-active reporter-labeled target molecule in order to generate probe-target complexes, and

(d) detecting an electrochemical signal in the pores of the porous substrate.

31. The method of Claim 30, wherein molecular interactions between an immobilized probe and an electrochemically active reporter-labeled target molecule are detected by applying an electrochemical detection method selected from the group of impedance, AC impedance, impedance spectroscopy, cyclic voltammetry, alternating cyclic voltammetry, stripping voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, and combinations thereof.

32. The method of Claim 31, wherein the electrochemical detection method is AC impedance and the AC impedance is measured over a range of frequencies prior to and after exposing the plurality of probes immobilized onto the surface of the pores of a porous substrate to a reaction mixture containing a target molecule.

33. The method of Claim 31, wherein the electrochemical detection method is AC impedance and the AC impedance is measured by transient methods with AC signal perturbation superimposed upon a DC potential applied to an electrochemical cell.

34. The method of Claim 31, wherein the electrochemical detection method is AC impedance and the AC impedance is measured by impedance analyzer, lock-in amplifier, AC bridge, AC voltammetry, or combinations thereof.

35. The method of Claim 31, wherein the molecular interactions detected thereby are single base mismatches within nucleic acid probe-target complexes.

36. The method of Claim 31, wherein the molecular interaction detected is quantification of electrochemically active reporter-labeled target molecules in a reaction mixture for gene expression analyses.

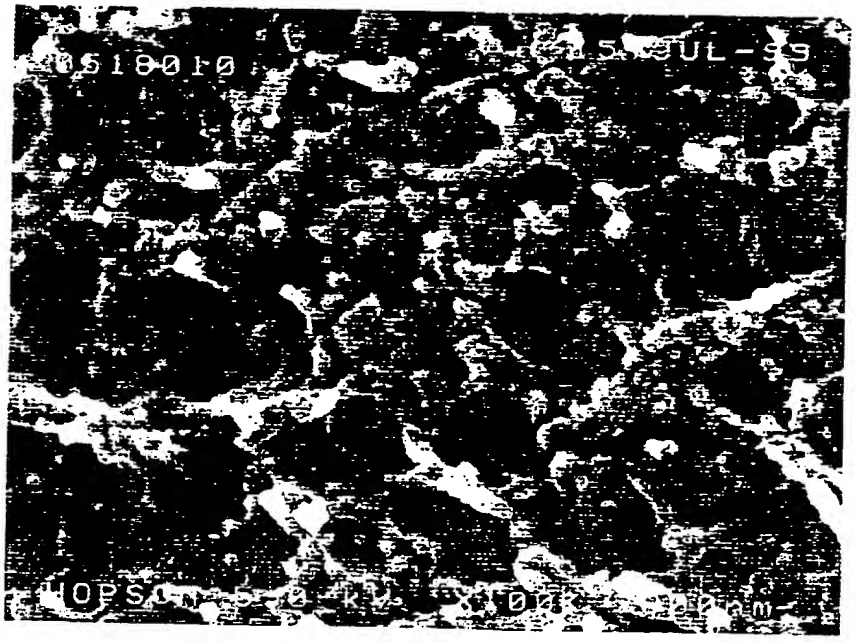
37. The method of Claim 31, wherein the electrochemically active reporter-labeled target molecules are labeled with electrochemical reporter groups comprising a transition metal complex.

38. The method of Claim 37, wherein the transition metal ion is ruthenium, cobalt, iron, or osmium.

39. The method of Claim 31, wherein the electrochemically active reporter-labeled target molecules are labeled with electrochemical reporter groups selected from the group consisting of 1,4-benzoquinone, ferrocene, tetracyanoquinodimethane, N,N,N',N'-tetramethyl-*p*-phenylenediamine, and tetrathiafulvalene.

40. The method of Claim 31, wherein the electrochemically active reporter-labeled target molecules are labeled with electrochemical reporter groups selected from the group consisting of 9-aminoacridine, acridine orange, aclarubicin, daunomycin, doxorubicin, pirarubicin, ethidium bromide, ethidium monoazide, chlortetracycline, tetracycline, minocycline, Hoechst 33258, Hoechst 33342, 7-aminoactinomycin D, Chromomycin A_3, mithramycin A, Vinblastine, Rifampicin, $Co(bipyridine)_3^{3+}$, Fe-bleomycin, and $Os(bipyridine)_2(dipyridophenazine)_2^{+}$.

FIG. 1



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/00421

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/543 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 226 470 A (UNILEVER PLC ;UNILEVER NV (NL)) 24 June 1987 (1987-06-24) claims column 2, line 21 - line 59 column 3, line 12 - line 22 column 5, line 4 - line 12 ---	1,4-16, 21-34 35-40
X Y	WO 96 28538 A (MESO SCALE TECHNOLOGIES LLC) 19 September 1996 (1996-09-19) the whole document ---	1-40 35-40
X	WO 92 21976 A (FISONS PLC) 10 December 1992 (1992-12-10) claims page 2, paragraph 3 -page 3, paragraph 1 page 4, paragraph 2 - paragraph 3 page 8, paragraph 2 --- -/--	1-40

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
1 June 2001	12/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Routledge, B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/00421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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